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(19) (CA) **CANADIAN PATENT** (12)

(54) Use of Synthetic Bifunctional Ligand for the Immunometric Determination of the Concentration Ratio of Two Solutes

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The Use of a Synthetic Bifunctional Ligand for the Immunometric Determination of the Concentration Ratio of Two Solutes

Introduction

The specification describes a simple technique (a kit) for determining reliably the fertile period of the human menstrual cycle. Such a kit will be suitable for home use, to enable a woman to determine when she is fertile and so avoid conception by periodic abstinence from intercourse. Conversely the ability to predict ovulation will be valuable in cases where conception is desired.

It is proposed to use a technique in which the ratio of two steroid metabolites, in urine, is measured directly; a signal being generated in direct proportion to the value of this ratio. The two metabolites are: 3-hydroxy-oestra-1,3,5 (10) - triene-17-one 3-β-D-glucupyranosiduronic acid (trivial name: oestrone-3-glucuronide or E13G) and 5β-pregnane-3α,20α-diol 3α-β-D-glucupyranosiduronic acid (trivial name: pregnanediol-3-glucuronide or PD3G). The signal will be a colour produced by an enzyme-labelled antibody. The novelty of the approach lies in the use of a mixed steroid glucuronide-protein immunocomplex to measure directly a steroid/steroid ratio. This principle can be applied to the measurement of any solute/solute ratio.

Materials

Mixed steroid glucuronide-protein complex.

The scheme is based on the preparation of a macromolecule containing both oestrone-glucuronide and pregnanediol-glucuronide covalently linked to a protein, such as bovine serum albumin (BSA):

(oestrone-3-glucuronide)$_x$-BSA-(pregnanediol-3-glucuronide)$_y$.

The method used will be the mixed anhydride reaction as described[1] with the difference that two steroids glucuronides instead of one would be involved in the reaction. The advantage of using a protein, such as bovine serum albumin is that the number of molecules of each steroid glucuronide per molecule of BSA can be independently varied by adjusting the stoichiometry of reagents. It is intended to synthesise a range of mixed steroid glucuronide-BSA complexes with varying ratios of oestrone-glucuronide to pregnanediol-glucuronide. Such mixed steroid glucuronide-BSA complexes will be capable of simultaneously binding both oestrone-glucuronide antibodies and pregnanediol-glucuronide antibodies. The complex with an optimum ratio will finally be selected after testing in the reaction scheme set out below.

Antisera

Immunoglobulin enriched fractions of both oestrone-3-glucuronide and pregnanediol-3α-glucuronide antisera will be used.

Oestrone glucuronide antiserum labelled with the enzyme "horse radish peroxidase" prepared by a modification of the method using glutaraldehydeas the cross linking reagent[2]. An alternative enzyme label alkaline phosphatase will also be investigated.

Solid Phase

Polystyrene test tubes (50 x 6mm) obtained clean and sterile from Sterilin Ltd will be used as a solid phase. The antibody coated tubes are robust and can be stored empty, in a sealed container, at -20°C for many months, without loss of antibody activity. Alternative solid phase supports will be investigated such as filter paper strips, to which antibody is covalently bound.

Procedure

The method proposed is in two parts. (1) preparation of a solid phase immunocomplex (Fig.1); (2) the testing of a sample for a E13G/PD3G ratio (Fig.2).

(1) Preparation of the solid phase immunocomplex

1. Coating of tubes with pregnanediol-glucuronide antiserum.
2. Buffer wash.
3. Incubation of antibody coated tubes with the mixed steroid glucuronide-BSA complex (ie E13G-BSA-PD3G).
4. Buffer wash.
5. Incubation of coated tubes with oestrone glucuronide antiserum.
6. Buffer wash.
7. Store empty, but sealed at -20°C until required.

(2) Use of above preparation to test for the E13G/PD3G

1. Add (diluted) urine to immunocomplex coated tubes. Incubate.
2. Buffer wash.
3. Add excess enzyme labelled oestrone-glucuronide antisera.
4. Buffer wash.
5. Measure amount of label, by addition of substrates followed by colorimetry.

NB: Excess reagent in step 3 will enable this to be a very short step. Also any non-specific interfering factors from the urine sample will be washed away therefore enzyme blanks should be minimal.

Rationale

The reaction end point will be determined by the amount of residual labelled oestrone glucuronide antibody bound to the solid phase, and this will be proportional to the number of exposed oestrone glucuronide residues on the solid phase after incubation with diluted urine. This in turn is dependent on two factors. (1) the number of

E13G residues will be proportional to the concentration of oestrogen glucuronide in the urine sample, since free oestrone glucuronide will competitively displace non-labelled oestrone glucuronide antibodies from the immunocomplex (2) the amount of immunocomplex bound to the solid phase will be inversely proportional to the concentration of pregnanediol glucuronide in the urine sample, as free pregnanediol glucuronide will competitively displace BSA linked preganediol glucuronide from the solid phase pregnanediol glucuronide antibodies.

It follows that, for samples containing high concentrations of oestrone glucuronide and low concentrations of pregnanediol glucuronide (periovulatory phase) maximum binding of labelled antibody will be ahieved i.e. high E13G/PD3G ratio will give a high signal.

Three other extreme conditions are possible:
(1) low oestrone glucuronide + low preganediol glucuronide (early follicular phase);
(2) low oestrone glucuronide + high pregnanediol glucuronide;
(3) high oestrone glucuronide + high pregnanediol glucuronide (mid luteal phase).

Under condition (1), unlabelled oestrone glucuronide antibodies will not be displaced from the immunocomplex, no labelled oestrone glucuronide antibodies can bind to the solid phase and a low signal will result. Under conditions (2) and (3) the immunocomplex will be cleaved from the solid phase altogether, no oestrogen residues will remain on the solid phase and no signal will be possible.

The above scheme has a number of advantages.
(1) Colour development occurs when the test is positive.
(2) Interfering factors from the urine sample are eliminated by washing after absorption onto solid phase.

(3) The test is independent from urine volume.
(4) The use of a solid phase technique enables the use of a simple "dipstick test".

Optimisation and evaluation of the kit

The kit will be optimised in terms of 'tuning' the colour signal response to correspond with the range of oestrone-3-glucuronide/pregnanediol-3-glucuronide ratios that occur in a normal menstrual cycle. The following variables will be investigated in order to achieve this end:
(a) Variation of the number of residues of oestrone-3-glucuronide and/or pregnanediol-3-glucuronide per molecule of mixed steroid-protein complex.
(b) Variation in the dilution of pregnanediol-3-glucuronide antiserum used to coat the solid phase.
(c) Variation in the characteristics of the unlabelled antisera, e.g. high versus low affinity antiserum.
(d) Variation in the characteristics of the enzyme-labelled antiserum.
(e) Variation in the nature of the enzyme label and/or substrates.

It is intended to examine urines from a large number of menstrual cycles using the kit. Using a simple colour chart, significant measures in colour intensity will be recorded and correlated with the fertile period.

Preliminary data on the E1PG/PD3G ratio from nine normal menstrual cycles by conventional analyses have shown that the ratio increases 2 to 5 fold from the mean follicular phase baseline value (days - 12 to - 6) to the pre-ovulatory peak value. Differences in chromophore concentrations of this magnitude are readily detected by the human eye therefore in theory the kit is feasible.

However it has been observed that the absolute value of the ratio can differ from woman to woman. For the nine subjects examined the follicular phase baseline ratio (mean of days -12 to - 6) ranges from 0.01 to 0.03 and the pre-ovulatory peak value (days - 2 to 0) ranges from 0.028 to 0.156. In terms of a kit this indicates that an absolute colour endpoint may not be suitable, rather a relative colour change would mark the end point.

It is envisaged that the kit would contain a colour chart, graduated in increasing intensities of colour. For each woman a follicular phase baseline of colour intensity would be recorded on the chart. As mid cycle approaches an increase in colour intensity by a certain number of graduations on the chart would be taken as a positive result.

In this specification the term "irreversible bond" means a bond, the dissociation constant of which is lower than the dissociation constant of an immunochemical bond. In particular the bonds between the solid phase and the second antibody have a lower dissociation constant than the dissociation constants of the immunochemical bonds which bind the first and second antibodies to the ligand molecules.

References

[1]Samarajeewa P & Kellis. A.E. (1975) Biochem.J.151,369-376

[2]Van Weemen B.& Schuurs.A. (1974) FEBS Letters 43,215.

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The embodiments of the invention in which an exclusive property of privilege is claimed are defined as follows:

1. A reagent system for use in determining the concentration ratio of a first antigenic solute to a second antigenic solute, comprising a first and a second component, the first component comprising;

a solid phase,

a first antibody capable of reversibly binding to the first antigenic solute,

a second antibody capable of reversibly binding to the second antigenic solute,

a ligand molecule comprising one or more molecules of the first antigenic solute and one or more molecules of the second antigenic solute covalently bound to a support molecule,

wherein the second antibody is irreversibly bound to the solid phase, the ligand molecule is capable of being reversibly bound to the solid phase by the reversible binding of the second antibody with one or more molecules of the second antigenic solute bound to the support molecule and the first antibody is capable of being reversibly bound to the ligand molecule by the reversible binding of the first antibody with one or more molecules of the first antigenic solute bound to the support molecule, thereby forming an immunocomplex bound to the solid phase,

and the second component comprising;

a labelled antibody having substantially the same antigenic specificity as the first antibody.

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2. A reagent system according to claim 1 wherein the support molecule is a protein.

3. A reagent system according to claim 2 wherein the protein is bovine serum albumin.

4. A reagent system according to claim 1, 2 or 3, wherein the first and second antibodies are immunoglobulin enriched fractions of antisera to the first and second antigens respectively.

5. A reagent system according to claim 1, 2 or 3 wherein the labelled antibody is labelled with an enzyme.

6. A reagent system according to claim 1, 2 or 3, wherein the enzyme labelled antibody is capable of producing a visible signal in conjunction with a substrate.

7. A reagent system according to claim 1, 2 or 3, wherein the labelled antibody is labelled with horseradish peroxidase.

8. A reagent system according to claim 1, 2 or 3, wherein the first and second antigenic solutes are steroids or steroid metabolites.

9. A reagent system according to claim 1, wherein the first antigenic solute is 3-hydroxy-oestra-1,3,5(10)-triene-17-one

3-β-D-glucupyranosiduronic acid (oestrone-3-glucuronide).

10. A reagent system according to claim 1, wherein the second antigenic solute is 5β-pregnane-3α, 20α-diol 3α-β-D-glucupyranosiduronic acid (pregnanediol-3-glucuronide).

11. A reagent system according to claim 9 or 10 when used in a method for the detection of the fertile period of the menstrual cycle.

12. A process for the determination of the concentration ratio of a first antigenic solute to a second antigenic solute using the reagent system according to claim 1, 2 or 3 comprising the steps of:

placing a sample containing a first and second antigenic solute in contact with the first component of the reagent system, thereby causing competitive dissociation of the immunocomplex, the equilibrium of which dissociation depends upon the ratio of the concentration of the first and second solutes present in the sample,

washing the first component with a buffer solution, thereby removing immunocomplexes not bound to the solid phase, placing the labelled antibody in contact with the first component thereby allowing the formation of an immunocomplex with non-complexed molecules of the first antigenic solute on the ligand molecule, washing the first component with a buffer solution, thereby removing immunocomplexes not bound to the solid phase, and measuring the amount of labelled antibody associated with the solid phase.

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13. A process for the determination of the concentration ratio of a first antigenic solute to a second antigenic solute using the reagent system according to claim 1, 2 or 3, wherein the first and second antibodies are immunoglobulin enriched fractions of antisera to the first and second antigens respectively and comprising the steps of:

placing a sample containing a first and second antigenic solute in contact with the first component of the reagent system, thereby causing competitive dissociation of the immunocomplex, the equilibrium of which dissociation depends upon the ratio of the concentration of the first and second solutes present in the sample,

washing the first component with a buffer solution, thereby removing immunocomplexes not bound to the solid phase, placing the labelled antibody in contact with the first component thereby allowing the formation of an immunocomplex with non-complexed molecules of the first antigenic solute on the ligand molecule, washing the first component with a buffer solution, thereby removing immunocomplexes not bound to the solid phase, and measuring the amount of labelled antibody associated with the solid phase.

14. A process for the determination of the concentration ratio of a first antigenic solute to a second antigenic solute using the reagent system according to claim 1, 2 or 3, wherein the labelled antibody is labelled with an enzyme and comprising the steps of:

placing a sample containing a first and second anti-

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genic solute in contact with the first component of the reagent system, thereby causing competitive dissociation of the immunocomplex, the equilibrium of which dissociation depends upon the ratio of the concentration of the first and second solutes present in the sample,

washing the first component with a buffer solution, thereby removing immunocomplexes not bound to the solid phase, placing the labelled antibody in contact with the first component thereby allowing the formation of an immunocomplex with non-complexed molecules of the first antigenic solute on the ligand molecule, washing the first component with a buffer solution, thereby removing immunocomplexes not bound to the solid phase, and measuring the amount of labelled antibody associated with the solid phase.

15. A process for the determination of the concentration ratio of a first antigenic solute to a second antigenic solute using the reagent system according to claim 1, 2 or 3, wherein the enzyme labelled antibody is capable of producing a visible signal in conjunction with a substrate and comprising the steps of:

placing a sample containing a first and second antigenic solute in contact with the first component of the reagent system, thereby causing competitive dissociation of the immunocomplex, the equilibrium of which dissociation depends upon the ratio of the concentration of the first and second solutes present in the sample,

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washing the first component with a buffer solution, thereby removing immunocomplexes not bound to the solid phase, placing the labelled antibody in contact with the first component thereby allowing the formation of an immunocomplex with non-complexed molecules of the first antigenic solute on the ligand molecule, washing the first component with a buffer solution, thereby removing immunocomplexes not bound to the solid phase, and measuring the amount of labelled antibody associated with the solid phase.

Figure 1:
Preparation of Mixed Steroid Glucuronide Immunocomplex on a Solid Phase

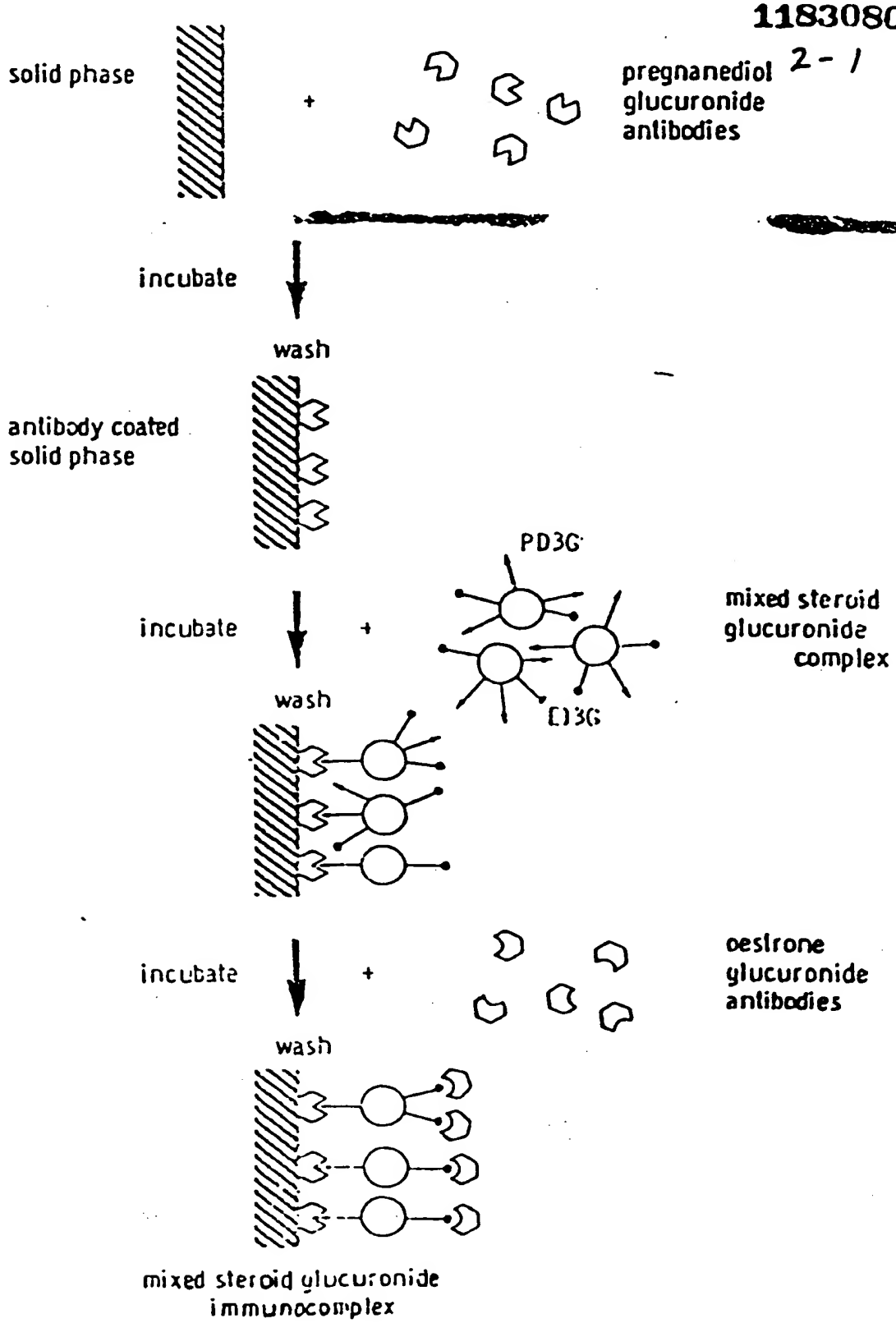

Figure 2:
Estimation of E13G/PD3G Ratio (four extreme situations are shown)